

Reproduction of Proventriculitis in Commercial and Specific-Pathogen-Free Broiler Chickens

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SUMMARY. Proventriculitis was studied by experimentally reproducing the disease in broiler chickens. One-day-old infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV) antibody positive commercial broilers and 1-day-old antibody negative specific-pathogen-free (SPF) broilers were orally gavaged with proventricular homogenates produced from the proventriculi of broilers with proventriculitis. At 7 and 14 days, both commercial and SPF broilers had enlargement of the proventriculus with necrosis of the glandular epithelium and lymphocytic infiltrates in the proventricular glands. SPF broilers exposed to the proventricular homogenates developed infectious bursal disease, and IBDV was detected by reverse transcriptase–polymerase chain reaction (RT-PCR). They also were positive by RT-PCR to IBV and developed nephritis. Commercial broilers developed mild nephritis but not bursal disease and were negative for IBDV and positive for IBV by RT-PCR. Both homogenate-inoculated commercial and SPF chickens were negative for reovirus and Newcastle disease virus by RT-PCR and variably positive for adenovirus by PCR. Bacteria were not identified in histologic sections, nor were they isolated from affected proventriculi. Indirect fluorescent antibody assay using convalescent sera detected intracytoplasmic staining in the proventricular glandular epithelial cells. Examination of thin sections of proventriculi using electron microscopy identified virus-like particles approximately 120 nm in diameter within the cytoplasm of these cells at 7 days after inoculation. Passage of proventricular homogenate filtrates in chicken embryos for virus isolation caused stunting, and allantoic fluid from these eggs was positive for IBV by RT-PCR.

RESUMEN. Reproducción de proventriculitis en pollos de engorde comerciales y libres de patógenos específicos.

Mediante la reproducción experimental de la enfermedad, se estudió la proventriculitis en pollos de engorde. Homogeneizados de proventrículo generados de los proventriculos de pollos de engorde con proventriculitis, fueron administrados con sonda por la vía oral, a pollos de engorde comerciales de un día de edad con anticuerpos contra el virus de la enfermedad infecciosa de la bolsa y virus de bronquitis infecciosa, así como a pollos de engorde libres de patógenos específicos negativos a anticuerpos. Tanto los pollos de engorde comerciales como los libres de patógenos específicos mostraron un aumento de tamaño del proventrículo con necrosis del epitelio glandular e infiltración linfocítica en las glándulas proventriculares a los 7 y 14 días posteriores a la inoculación. Los pollos de engorde libres de patógenos específicos expuestos a los homogeneizados de proventrículo, desarrollaron enfermedad infecciosa de la bolsa detectándose a través de reacción en cadena por la polimerasa–transcriptasa reversa (por sus siglas in Inglés RT-PCR). A su vez, estas aves resultaron positivas a la prueba RT-PCR para bronquitis infecciosa y desarrollaron nefritis. Los pollos de engorde comerciales desarrollaron nefritis leve, pero no enfermedad infecciosa de la bolsa, resultando negativos para el virus de la enfermedad infecciosa de la bolsa y positivos para bronquitis infecciosa mediante la prueba RT-PCR. Tanto los pollos de engorde comerciales como los libres de patógenos específicos resultaron negativos para reovirus y el virus de la enfermedad de Newcastle por medio de la prueba RT-PCR, mientras que para adenovirus la positividad fue variable a la prueba de PCR. No fue posible la identificación de bacterias en los cortes histológicos ni su aislamiento de los proventriculos afectados. Se detectó tinción intracitoplasmática de las células pertenecientes al epitelio glandular del proventrículo con la prueba de inmunofluorescencia indirecta para anticuerpos utilizando suero de aves convalecientes. La evaluación de cortes de proventrículo a través de microscopía electrónica permitió la identificación de partículas parecidas a virus de aproximadamente 120 nm de diámetro dentro del citoplasma de estas células 7 días posteriores a la inoculación. El pasaje de homogeneizados de proventrículo en embriones de pollo para aislamiento viral causó enanismo y el fluido alantoideo de estos embriones resultó positivo al virus de bronquitis infecciosa mediante la prueba de RT-PCR.

Key words: proventriculitis, broilers, infectious bursal disease virus, infectious bronchitis virus

Abbreviations: CAM = chorioallantoic membrane; dpi = days postinoculation; EM = electron microscopy; FITC = fluorescein isothiocyanate; H&E = hematoxylin and eosin; IBDV = infectious bursal disease virus; IBV = infectious bronchitis virus; IFA = indirect fluorescent antibody assay; MG = *Mycoplasma gallisepticum*; MS = *Mycoplasma synoviae*; NDV = Newcastle disease virus; PBS = phosphate-buffered saline; –PV = negative proventricular homogenate; RFLP = restriction fragment length polymorphism; SPF = specific pathogen free

Proventriculitis is an infectious disease of chickens of unknown etiology. It is characterized by an enlarged, atonic proventriculus that is filled with fluid and feed (2,7,11). The gastric isthmus connecting the proventriculus and gizzard is dilated and weakened.

The poultry industry reports sporadic, though economically important, outbreaks of proventriculitis in broilers (2). The main

economic impact of proventriculitis is due to condemnation of contaminated carcasses subsequent to the rupture of the proventriculus during routine evisceration (2,11). Proventriculitis is more severe in younger birds (4–5 wk of age) and has been associated with impaired growth, poor feed conversion, intestinal fragility, stunting syndrome, and passage of undigested feed (1,3,11,16,21,28).

Routes of natural infection of proventriculitis are not known; however, chickens can be infected experimentally by oral inoculation with homogenized proventriculi from affected chickens (2,11,23,24).

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Because the disease is also reproduced with proventricular homogenate filtrates (0.2 μm), a virus is suspected as the etiologic agent (2,11). Consequently, the disease is also termed transmissible viral proventriculitis (7). However, the lesions and the effects on production are more severe in birds treated with unfiltered homogenates, suggesting an additive effect due to larger infectious agents (11).

Potential infectious causes of proventriculitis include adenovirus (16,18), reovirus (17,18,21), infectious bronchitis virus (a coronavirus, IBV) (31), infectious bursal disease virus (a birnavirus, IBDV) (2,11,12,20,28), and megabacterium (9,10,19,25). However, none of these agents have been found in a majority of cases. Electron microscopy has detected adenovirus-like viral particles in acute lesions, but isolation of this virus from affected proventriculi has been unsuccessful (7,11).

The objective of this study was to reproduce proventriculitis in broiler chickens, characterize the changes present in the proventriculus and other organs, and examine the affected proventriculus for the presence of virus or bacteria by histologic, bacteriologic, virologic, and molecular methods.

MATERIALS AND METHODS

Chickens. One-day-old unvaccinated broiler chicks were obtained from a commercial hatchery. Also, fertile white Plymouth Rock chicken eggs (Southeast Poultry Research Laboratory, U.S. Department of Agriculture, Athens, GA) were obtained from a breeder flock maintained under SPF conditions and hatched. The parent flock and all progeny were free of common poultry diseases, specifically IBDV, Marek's disease virus, IBV, reovirus, and chicken anemia virus. All chicks were wing banded, weighed, separated into groups, and maintained in positive pressure Horsfall isolation units. Feed and water were provided *ad libitum*.

Proventricular homogenates. Two different proventricular homogenates were used. Homogenate 1 (Hom. 1) was prepared from proventriculi from 4-wk-old chickens with proventriculitis, obtained from a commercial Cornish hen processing plant in northwest Arkansas (2). Homogenate 2 (Hom. 2) was prepared from proventriculi of broiler chickens that developed proventriculitis after being challenged at 1 day of age with Hom. 1 (11).

Experimental design. Eighteen 1-day-old commercial broilers and 18 one-day-old SPF broilers were each divided into three equal groups. The first group of each type was inoculated by oral gavage with 1 ml of sterile saline solution (negative control). The second group received 1 ml of proventricular homogenate 1 (Hom. 1). The third group received 1 ml of proventricular homogenate 2 (Hom. 2).

Sample collection and processing. At 7 and 14 days of age, three birds from each group were examined, weighed, bled, killed by cervical dislocation, and necropsied. Bursa, proventriculus, spleen, and all the right lobes of the thymus were weighed, and portions of these organs and of liver, kidney, duodenum, pancreas, heart, gizzard, and bone were collected from each bird and fixed immediately by immersion in 10% neutral buffered formalin for 24 hr. Tissues were then processed and embedded in paraffin using routine histologic techniques. An additional sample of the proventriculi was collected in 2% glutaraldehyde, 2% paraformaldehyde, 0.2% picric acid, and 0.1 M cacodylate buffer at pH 7.2–7.3 for thin sectioning and electron microscopic examination. The remaining portions of proventriculi were pooled within groups and collected in sterile plastic tubes on ice, and homogenates were prepared as previously described (2,11). Briefly, proventriculi were washed in sterile phosphate-buffered saline (PBS) three times on a magnetic stirrer to remove feed residues and toxins. Washed proventriculi were then diluted 1:1 w/v in sterile PBS and homogenized with a sterile commercial blender (Waring, New Hartford, CT). Homogenates were then frozen at -80°C . Relative organ weights were obtained using the formula (relative organ weight = [organ weight/body weight] \times 100).

Histopathology. Paraffin-embedded tissues were sectioned, mounted, and stained using hematoxylin and eosin (H&E), and examined for lesions blinded as to treatment using light microscopy.

Each tissue section of proventriculus, bursa, thymus, and spleen was also assigned a lesion severity score. A lesion score of 1 represented no lesions. For bursal sections, 2 was defined as mild variation in follicle size, 3 as moderate variation in size of follicles, and 4 as either necrosis or follicle atrophy. For proventricular sections, 2 was defined as mild glandular luminal ectasia, 3 as glandular ectasia plus mild glandular necrosis and lymphoid infiltrates in the interglandular interstitium, and 4 as either acute glandular necrosis or severe fibrosis with lymphoid infiltrates. For thymus sections, 2 was defined as mild cortical thinning, 3 as moderate cortical thinning, and 4 as absence of cortical lymphocytes. For spleen sections, 2 was defined as mild lymphocyte depletion, 3 as moderate lymphocyte depletion, and 4 as severe lymphocyte depletion.

For identification of bacteria by light microscopy, tissue sections of proventriculi were stained by the Warthin-Starry technique (4) and a modified *Helicobacter pylori* and gastric stain (6).

Serology. Serum samples obtained at 7 and 14 days of age, from both commercial and SPF broilers, were examined for antibody to IBDV, IBV, Newcastle disease virus (NDV), reovirus, *Mycoplasma synoviae* (MS), and *Mycoplasma gallisepticum* (MG), using commercially available enzyme-linked immunosorbent assay tests (Idexx Laboratories, Inc. Westbrook, ME).

Bacteriology. Pooled proventricular homogenates from experimentally infected birds from each group were diluted in sterile saline and plated on Campy blood agar (Remel, Lenexa, TX). Inoculated plates were placed into gaspak pouches (BD Diagnostic Systems; Sparks, MD) and incubated at 42°C for 48 hr. To check for anaerobic growth, blood agar and phenylethyl alcohol agar plates were streaked and incubated overnight at 37°C in a gaspak pouch. The proventricular homogenates were also plated on Sabouraud dextrose agar plates and incubated at 37°C overnight, and then maintained at room temperature and examined daily for mold growth. Isolation of *Salmonella* sp. was attempted by standard protocol using tetrathionate enrichment broth (8).

Virus isolation. Three samples were examined by virus isolation: proventricular homogenate 2 (Hom. 2), proventricular homogenate made from pooled proventriculi obtained from commercial chickens challenged with Hom. 2 (Hom. 2 com) at 7 days postinoculation (dpi), and negative proventricular homogenate from chickens in a control group (–PV). Each was frozen and thawed three times; sediment was removed by centrifugation ($2500 \times g$ for 30 min at 4°C); and the supernatants were forced through a series of glass fiber filters with a final membrane pore size of 0.2 μm . Four groups of five SPF leghorn chicken embryos were inoculated at 9 days of age via chorioallantoic membrane and allantoic cavity routes (26), with 0.2 ml of one of the following: Hom. 2 filtrate, Hom. 2 com filtrate, –PV filtrate, and sterile saline. Eggs were examined daily for embryo death. At 7 days postinoculation chorioallantoic membranes (CAMs) and allantoic fluid were aseptically collected and placed in sterile microfuge tubes and frozen at -80°C . A portion of each CAM was also collected in 10% buffered formalin and processed for histopathology. Five blind passes were done and at each, allantoic fluid and CAMs were diluted 1:10 in antibiotic diluent (with Gentamycin and Fungizone) prior to reinoculation.

RNA extraction. RNA was extracted from formalin-fixed paraffin-embedded bursas and proventriculi and from Hom. 1, Hom. 2, pooled proventricular homogenates from experimental groups at 7 dpi, and allantoic fluid from eggs inoculated with homogenate filtrates (fifth passage). Sections totaling 50 μm in thickness were cut from each formalin-fixed paraffin-embedded tissue block with a microtome and a new blade for each block. Sections were then deparaffinized (HemoDe and 100% ethanol; Fisher Scientific, Pittsburgh, PA). All tissues were digested with 10% proteinase K (Sigma Chemical Co., St. Louis, MO) for 1 hr at 50°C . RNA was extracted with Trizol (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's recommendations, diluted in 90% dimethyl sulfoxide, and frozen at -80°C until assayed.

DNA extraction. DNA was extracted from Hom. 1, Hom. 2, pooled proventricular homogenates from experimental groups at 7 dpi, and allantoic fluid from eggs inoculated with homogenate filtrates (fifth passage) using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer's recommendations. Extracted DNA was frozen at -80°C until assayed.

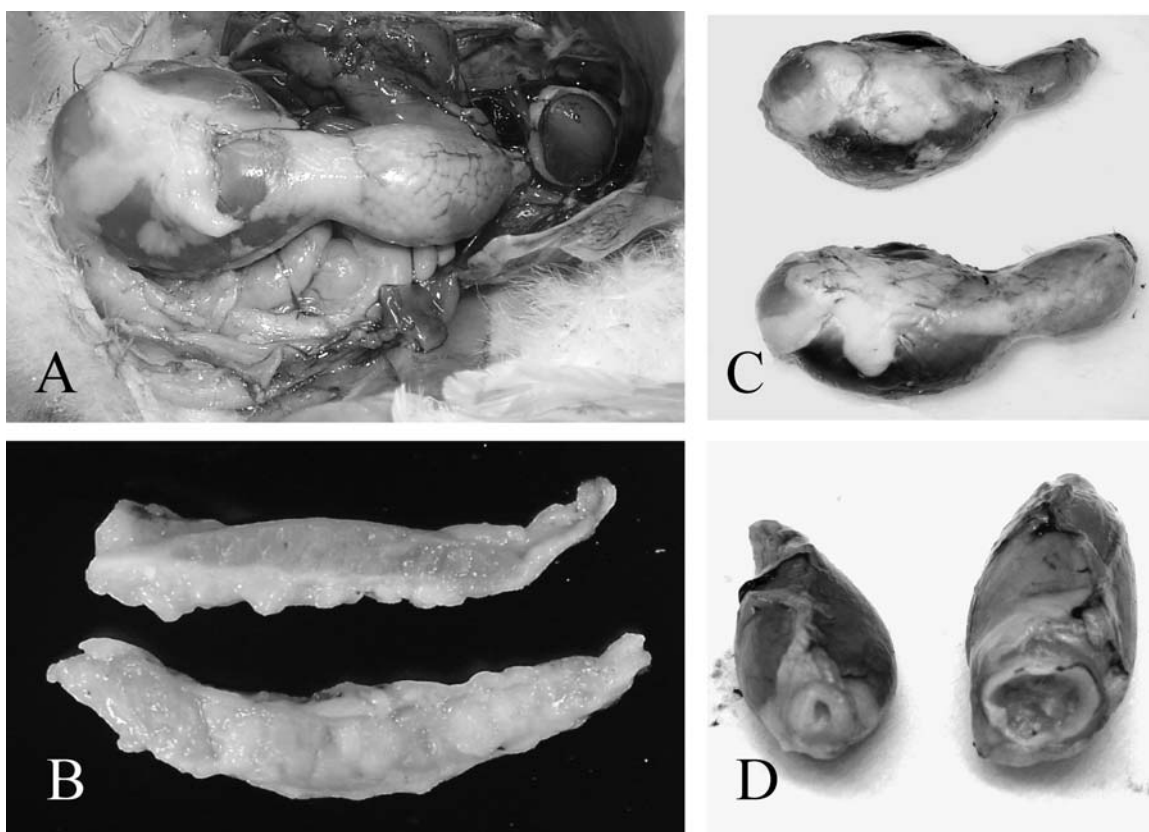


Fig. 1. (A) Proventriculitis in a commercial chicken inoculated with an infectious proventricular homogenate at 1 day of age (14 dpi). (B) Comparison between the proventricular wall of a normal chicken (upper section), and the proventricular wall of a chicken with proventriculitis (lower section) where the wall is thickened, with a white lobular pattern. (C and D) Proventriculi of a normal chicken (upper and on left) and a chicken with proventriculitis (lower and on right). The proventriculus is enlarged and the gastric isthmus distended in proventriculitis.

Real time reverse transcriptase–polymerase reaction (RT-PCR). For detection of IBDV, IBV, NDV, and reovirus, extracted RNA was denatured at 95 °C for 5 min and put on ice (only for IBDV and reovirus reactions). Real time RT-PCR was performed separately for each virus in each sample with reagents from the light cycler-RNA amplification Sybr® green I kit (Roche Molecular Biochemicals, Indianapolis, IN). The primers used were specific for amplification of each of the viruses (13,15,22,31). Amplification and detection of specific products was performed with a light cycler according to the manufacturer's recommendations (Roche light cycler version 3.0; Roche Molecular Biochemicals). Briefly, reverse transcription was done at 55 °C for 10 min, followed by denaturation at 95 °C for 30 sec. Forty PCR cycles were done consisting of denaturation (95 °C for 1 sec), hybridization (55 °C for 10 sec), and extension (72 °C for 13 sec). A melting curve analysis was done after an initial denaturation at 95 °C. The melting curve was es-

tablished with an initial temperature of 65 °C for 10 sec and then gradual temperature increase of 0.1 °C per second until reaching 95 °C. The melting temperature was used to confirm the identity of viral specific products obtained using real time RT-PCR. Additional confirmation of specific amplification was done by gel electrophoresis of 8 µl of the PCR products on 2% agarose (Sigma Chemical Co.) followed by ethidium bromide staining. PCR products from IBDV positive samples were purified using the QIAgen purification kit and sequenced (Molecular Genetics Instrumentation Facility; University of Georgia, GA). Sequence data were then analyzed by DNASTAR and sequences compared with that of known IBDV. Samples positive for IBV were analyzed by RT-PCR restriction fragment length polymorphism (RFLP) for molecular grouping (11).

PCR. PCRs for detection of adenovirus were performed with ready to go PCR beads (Pharmacia Biotech, Amersham Biosciences Corp.,

Table 1. Body weight gain, relative organ weights (percentage of body weight) and organ lesion scores of commercial broilers orally challenged at 1 day of age with sterile saline, proventricular homogenate 1 (Hom. 1), or proventricular homogenate 2 (Hom. 2), and necropsied at 7 or 14 days postinoculation (dpi).^A

dpi	Treatment	Body weight gain (g)	Proventriculus relative weight	Proventriculus lesion score	Bursa relative weight	Bursa lesion score	Thymus relative weight	Thymus lesion score	Spleen relative weight	Spleen lesion score
7	Saline	120 ± 10 ^a	0.81 ± 0.09 ^a	1.33 ± 0.57 ^a	0.10 ± 0.005 ^a	2.00 ^a	0.10 ± 0.02 ^a	1.00 ^a	0.02 ± 0.005 ^a	2.00 ^a
	Hom. 1	122 ± 6 ^a	1.20 ± 0.01 ^{ab}	3.00 ± 1.0 ^b	0.20 ± 0.04 ^a	3.00 ^a	0.23 ± 0.04 ^b	1.00 ^a	0.12 ± 0.03 ^b	2.00 ^a
	Hom. 2	98 ± 16 ^a	1.48 ± 0.33 ^b	3.66 ± 0.57 ^b	0.17 ± 0.06 ^a	3.0 ± 1.0 ^a	0.16 ± 0.65 ^{ab}	1.00 ^a	0.17 ± 0.02 ^b	2.00 ^a
14	Saline	399 ± 50 ^a	0.53 ± 0.05 ^a	1.33 ± 0.57 ^a	0.15 ± 0.03 ^a	1.33 ± 0.5 ^{ab}	0.20 ± 0.03 ^a	1.00 ^a	0.05 ± 0.005 ^a	1.66 ± 0.57 ^a
	Hom. 1	336 ± 2 ^a	1.06 ± 0.37 ^b	3.00 ± 1 ^b	0.23 ± 0.03 ^a	1.00 ^a	0.23 ± 0.03 ^a	1.00 ^a	0.12 ± 0.03 ^b	1.66 ± 0.57 ^a
	Hom. 2	402 ± 47 ^a	0.97 ± 0.17 ^b	4.00 ^b	0.15 ± 0.03 ^a	2.00 ^b	0.26 ± 0.07 ^a	1.00 ^a	0.08 ± 0.08 ^{ab}	1.00 ^a

^AMeans within a column and a time point with different lowercase superscripts are significantly different ($P < 0.05$). Means calculated from three birds in each group.

Table 2. Body weight gain, relative organ weights (percentage of body weight), and organ lesion scores of SPF broilers orally challenged at 1 day of age with sterile saline, proventricular homogenate 1 (Hom. 1), or proventricular homogenate 2 (Hom. 2), and necropsied at 7 or 14 days postinoculation (dpi).^A

dpi	Treatment	Body weight gain (g)	Proventriculus relative weight	Proventriculus lesion score	Bursa relative weight	Bursa lesion score	Thymus relative weight	Thymus lesion score	Spleen relative weight	Spleen lesion score
7	Saline	41 ± 3 ^a	0.93 ± 0.12 ^a	1.00 ^a	0.22 ± 0.04 ^a	2.66 ± 0.57 ^a	0.20 ± 0.05 ^a	1.00 ^a	0.25 ± 0.64 ^a	2.00 ^a
	Hom. 1	24 ± 7 ^b	1.29 ± 0.18 ^a	1.33 ± 0.5 ^a	0.08 ± 0.02 ^b	4.00 ^b	0.13 ± 0.005 ^a	2.66 ± 0.57 ^a	0.20 ± 0.06 ^a	2.00 ^a
	Hom. 2	22 ± 8 ^b	1.47 ± 0.46 ^a	3.00 ± 1.73 ^a	0.21 ± 0.02 ^a	3.33 ± 0.5 ^{ab}	0.18 ± 0.007 ^a	2.00 ± 1.73 ^a	0.21 ± 0.06 ^a	2.00 ^a
14	Saline	126 ± 19 ^a	0.72 ± 0.06 ^a	1.00 ^a	0.36 ± 0.12 ^a	1.33 ± 0.57 ^a	0.35 ± 0.19 ^a	1.00 ^a	0.27 ± 0.08 ^a	1.66 ± 0.57 ^a
	Hom. 1	88 ± 18 ^{ab}	0.98 ± 0.4 ^{ab}	2.00 ± 1 ^{ab}	0.11 ± 0.03 ^b	4.00 ^b	0.17 ± 0.07 ^b	3.00 ± 1.7 ^{ab}	0.17 ± 0.06 ^a	2.00 ^a
	Hom. 2	53 ± 9 ^b	1.55 ± 0.49 ^b	3.33 ± 1.1 ^b	0.16 ± 0.04 ^{ab}	3.33 ± 1.5 ^b	0.07 ± 0.03 ^b	4.00 ^b	0.14 ± 0.08 ^a	2.00 ^a

^AMeans within a column and a time point with different lowercase superscripts are significantly different ($P < 0.05$). Means calculated from three birds in each group.

Piscataway, NJ) and following the protocol from Raue *et al.* (26). An 8- μ l aliquot of each reaction was separated by electrophoresis in a 2% agarose gel (Sigma Chemical Co.) followed by ethidium bromide staining and examination with an ultraviolet transilluminator.

Indirect fluorescent antibody assay (IFA). All procedures were done at room temperature. Proventriculi tissues examined belonged to the inoculated commercial and SPF chickens collected at 7 dpi. Tissue sections (4 μ m thick) were cut from paraffin-embedded proventriculi and mounted on charged glass slides (Superfrost/Plus; Fisher Scientific). Paraffin was melted from slides (10 min at 65 °C) and removed by immersion in Hemo-De three times (5 min each). Slides were then air dried and digested with 10% proteinase K (Dako, Carpinteria, CA) for 5 min to expose antigenic target sites. IFA was performed on proventriculi sections using as primary antibody convalescent sera from SPF chickens at 14 dpi diluted 1:100 in sterile PBS. Slides were incubated for 20 min followed by three washes with PBS for 5 min each. A secondary antibody goat anti-chicken IgG fluorescein isocyanate (FITC) conjugated (KPL, Inc. Gaithersburg, MD) was used at a 1:40 dilution. Slides were incubated for 20 min and then washed three times with PBS. Slides were mounted using 1:1 glycerol/PBS and examined using a fluorescent microscope.

Electron microscopy (EM). Sections of proventriculus collected at 7 dpi, from one commercial and one SPF chicken inoculated with Hom. 1, from one commercial and one SPF chicken inoculated with Hom. 2, and from two controls, were sent for direct examination using a JEOL JEM 1210 transmission electron microscope (JEOL, Ltd., Peabody, MA) along with allantoic fluid collected from the fifth passage in eggs of Hom. 2, Hom. 2 com and -PV.

Statistical analysis. The body weight gain, relative organ weight, and lesion scores were analyzed using analysis of variance and means comparisons for all pairs using Tukey-Kramer's honestly significant difference (JMP software, SAS Institute Inc., Cary, NC). Significance was assumed at the 0.05 level of probability.

RESULTS

Clinical signs and macroscopic lesions. No clinical signs were observed in the control groups given saline or the commercial chickens given proventricular homogenates. SPF broilers given the homogenates were mildly depressed. Gross lesions were observed in all proventriculi from homogenate-inoculated commercial and SPF chickens. At both 7 and 14 days postinoculation, the proventriculi were enlarged, mottled, and had a dilated gastric isthmus (Fig. 1). The proventricular wall was thickened, with a mural white lobular pattern. These changes were more severe in the commercial broilers compared with SPF broilers and at 14 days postinoculation for both groups. No macroscopic lesions were observed in any other organs of the experimentally exposed birds.

Body weight gain. Commercial broilers inoculated with proventricular homogenate had no significant suppression of weight gain compared with age-matched control birds (Table 1). Weight gain in SPF broilers was significantly decreased by Hom. 1 and Hom. 2 at 7 dpi and by Hom. 2 at 14 dpi (Table 2).

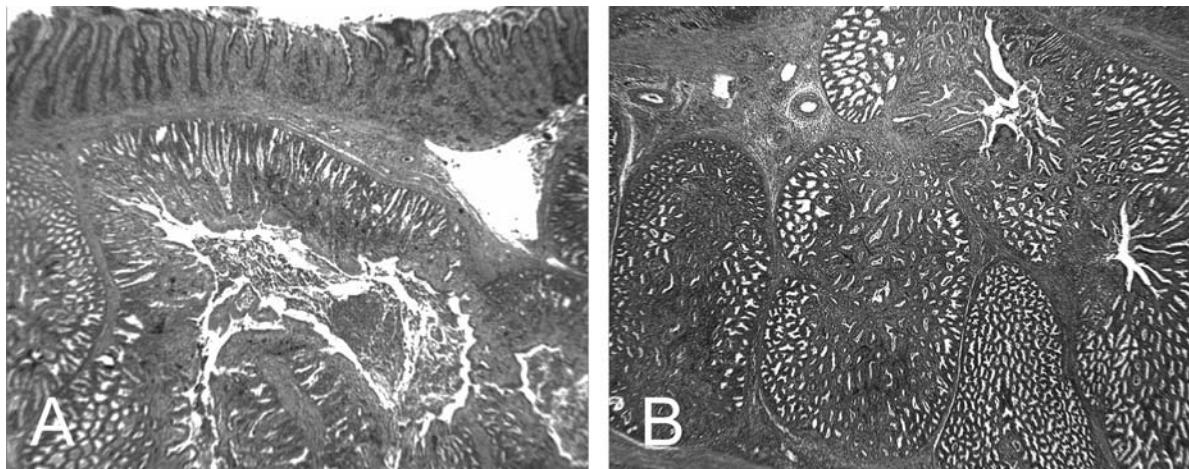


Fig. 2. Photomicrographs of proventriculi from a chicken with proventriculitis. (A) Degeneration and necrosis of glandular epithelium with coalescing of glands and lymphocytic infiltration in mucosa and glands (7 dpi). (B) Lymphocytic infiltration in the glandular interstitium with ductal epithelial hyperplasia (14 dpi). H&E.

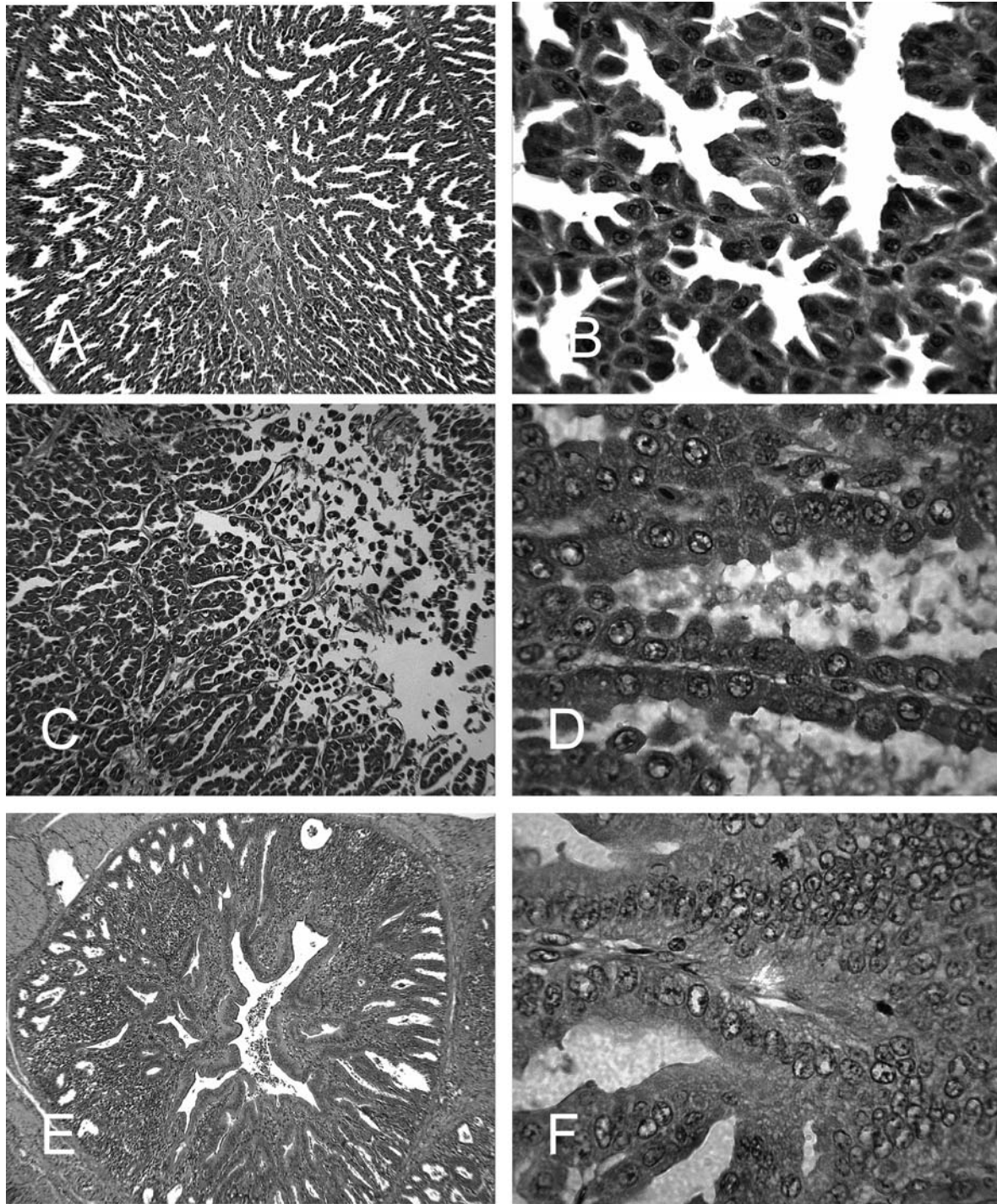


Fig. 3. Photomicrographs of proventriculi from a normal chicken (A and B) and from chickens with proventriculitis (C, D, E, and F). (C) Dilation of glandular sinus with separation of epithelial cells from basement membrane. (D) Nuclei of affected glandular epithelial cells are enlarged and pale with margined chromatin. (E) Columnar ductal epithelium replacing secretory glandular epithelium, with lymphocytic infiltration in the proventricular gland. (F) Hypertrophy and hyperplasia of ductal epithelium. H&E.

Organ weights and microscopic lesions. Commercial and SPF chickens that received Hom. 1 or Hom. 2 had increased proventricular organ/weight ratio, and microscopic lesions in their proventriculi at 7 and 14 dpi. The increase in proventriculi organ/weight ratio was significant in commercial broilers that received Hom. 2 at both 7 and 14 dpi. This increase was also significant in commercial chickens that received Hom. 1 and in SPF chickens that received Hom. 2, at 14 dpi. Proventricular lesion score was sig-

nificantly increased in the commercial chickens that received either one of the proventricular homogenates, at both 7 and 14 dpi. In SPF chickens, the increase in proventricular lesion scores was significant at 14 dpi in birds that received Hom. 2. Bursa and thymus organ/weight ratios were not affected in commercial broilers, but their spleen organ weight increased with both treatments (Table 1). SPF broilers that received Hom. 1 or Hom. 2 had smaller bursas and thymuses compared with controls at 14 dpi (Table 2). The decrease in size of

Table 3. RT-PCR and PCR results from proventricular homogenate and allantoic fluid samples. Proventricular homogenates used for inoculation of chickens (Hom. 1 and 2), proventricular homogenates obtained from chickens inoculated with saline, Hom. 1, or Hom. 2; from commercial (com) and SPF broilers at 7 dpi; allantoic fluid from embryos inoculated with Hom. 2. Filtrate, proventricular homogenate filtrate obtained from the proventriculi of commercial broilers inoculated with Hom. 2, and from proventricular homogenate filtrate made from the proventriculi of control chickens (–PV), harvested at the fifth pass.

Sample	IBDV	Reovirus	NDV	IBV	Adenovirus
Hom. 1	+	–	–	+	+
Hom. 2	+	–	–	+	–
Saline com 7 dpi	–	–	–	–	–
Hom. 1 com 7 dpi	–	–	–	+	+
Hom. 2 com 7 dpi	–	–	–	+	–
Saline SPF 7 dpi	–	–	–	–	–
Hom. 1 SPF 7 dpi	+	–	–	+	+
Hom. 2 SPF 7 dpi	+	–	–	+	–
AF Hom. 2 fifth pass	–	–	–	+	–
AF Hom. com 2 fifth pass	–	–	–	+	–
AF –PV fifth pass	–	–	–	–	–

the bursa and increase in bursa lesion score was significant at both 7 and 14 dpi in SPF chickens that received Hom. 1. SPF chickens that received Hom. 2 had a significant increase in bursa lesion score at 14 dpi. There was no difference in spleen relative weight and lesion score in SPF chickens.

Microscopically, at 7 dpi, acute necrosis of the proventricular glandular epithelium was present in both commercial and SPF chickens (Figs. 2 and 3). Collecting sinuses of the proventricular glands were dilated and contained desquamated epithelium, and severely affected glands coalesced. Nuclei of glandular epithelial cells were large and pale, with marginated chromatin. Lymphocytic infiltrates were present in the lamina propria of the mucosa and in the glandular interstitium in areas containing affected glandular epithelial cells. At 14 dpi, hyperplastic and hypertrophic columnar cells lined glandular primary, secondary, and tertiary ducts. Cuboidal to low columnar, pale, basophilic, and distinctly vacuolated duct-like epithelia replaced the destroyed alveolar secretory cells. Lymphoid germinal center formation was present in the glands and mucosa. There were no differences in lesion scores for bursas, thymuses or spleens between commercial chickens and controls. Bursas and

thymuses of SPF chickens that received either homogenate had increased lesion scores compared to controls at both 7 and 14 dpi.

Mild lymphocytic infiltrates were present in the intestine, pancreas, and liver of commercial and SPF chickens inoculated with Hom. 1 or Hom. 2, at both 7 and 14 dpi. All homogenate-inoculated SPF broilers also had moderate to severe lymphocytic infiltrates in their kidneys at both 7 and 14 dpi. Mild renal lymphocytic infiltrates were also present in commercial broilers inoculated with Hom. 1 or Hom. 2 at 14 dpi. No other lesions were present in the other organs examined from either the commercial or the SPF chickens.

Serology. Commercial broilers that received Hom. 1 or Hom. 2 were negative for reovirus, NDV, MG, and MS at 7 and 14 dpi. These birds were positive for IBDV and IBV at both time points. SPF broilers that received these proventricular homogenates were negative for IBDV, IBV, reovirus, NDV, MG, and MS at 7 dpi, but at 14 dpi seroconverted to IBDV and IBV.

Bacteriology. No bacteria were isolated from proventricular homogenates produced from birds experimentally infected by the methods described above. Also, no bacteria were observed by direct light microscopy in routine or special-stained sections.

Virus isolation. Embryos inoculated with either Hom. 2 or proventricular homogenate from commercial chickens inoculated with Hom. 2 (Hom. 2 com) were stunted in the second through fifth passages. Chorioallantoic membranes (CAMs) harvested from these eggs did not have plaques or lesions observed histopathologically.

RT-PCR and PCR. IBDV RT-PCR on paraffin-embedded tissues. Bursas and proventriculi of commercial broilers were all negative for IBDV. All bursas and some of the proventriculi of SPF broilers that received either Hom. 1 or 2 were positive for IBDV.

RT-PCRs and PCRs on proventricular homogenates and allantoic fluids. All samples were negative for reovirus and NDV (Table 3). Hom. 1 was positive for IBDV, IBV, and adenovirus. Hom. 2 was positive for IBDV and IBV and negative for adenovirus. Proventricular homogenates from commercial broilers inoculated with Hom. 1, and collected at 7 dpi, were negative for IBDV and positive for IBV and adenovirus. Proventricular homogenate from commercial broilers inoculated with Hom. 2 were negative for IBDV and adenovirus and positive for IBV. SPF broilers inoculated with Hom. 1 were positive for IBDV, IBV, and adenovirus and negative for other viruses. SPF broilers inoculated with Hom. 2 were positive for IBDV and IBV and negative for adenovirus. Allantoic fluids from embryos inoculated with Hom. 2 or Hom. 2 com were positive for IBV only.

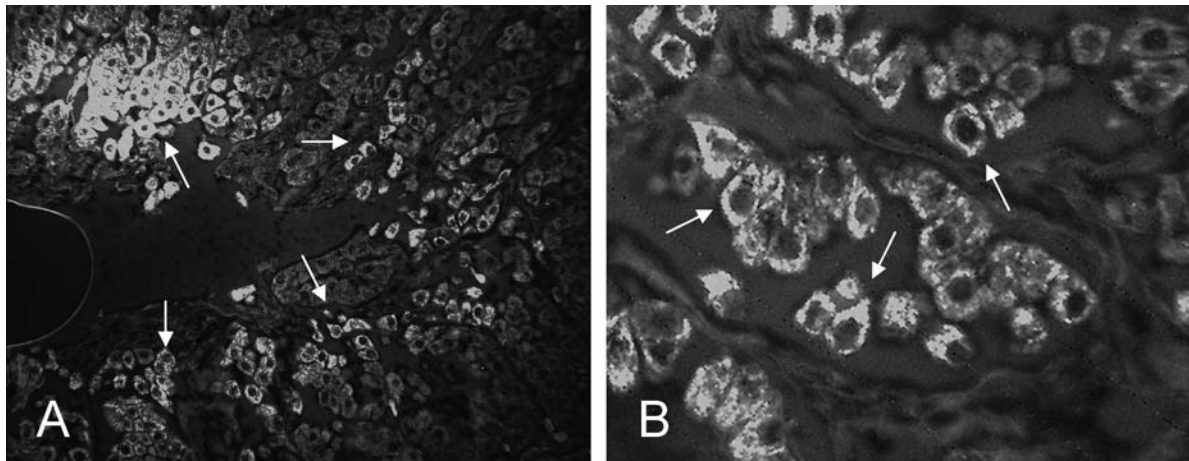


Fig. 4. (A) Positive immunochemical staining of proventricular epithelial cells of a chicken inoculated with a positive proventricular homogenate (7 dpi) (arrows). (B) Fluorescent staining localized in the cytoplasm of affected cells (arrows). Immunohistochemical staining was performed using convalescent sera as the primary antibody and goat anti-chicken IgG FITC conjugated as the secondary antibody.

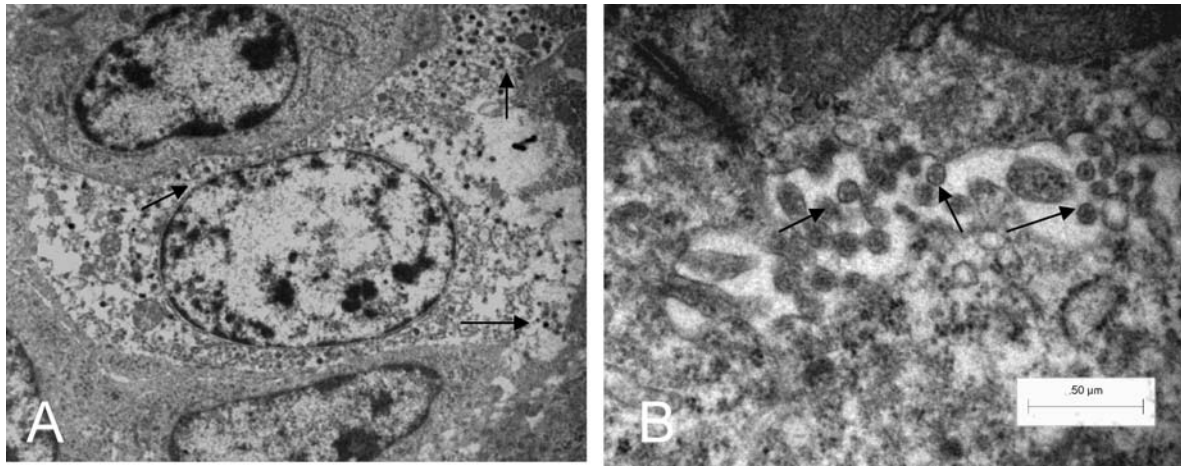


Fig. 5. (A) Proventricular glandular epithelial cell containing virus-like particles in the cytoplasm (arrows). (B) A higher magnification showing pleomorphic virus-like particles of approximately 100–120 nm in diameter budding at membranes of the endoplasmic reticulum and in smooth-walled vesicles in the cytoplasm of affected cells (arrows).

Molecular characterization of detected IBDV and IBV. Analysis of the sequence data obtained from the amplified IBDV revealed that this virus is an IBDV variant strain and was most similar to variant A. RFLP analysis of the amplified IBV determined that this virus strain was similar to the Connecticut strain.

Indirect fluorescent antibody assay. Positive staining was present in the cytoplasm of the glandular epithelial cells of all proventriculi from chickens inoculated with Hom. 1 and Hom. 2 examined at 7 dpi (Fig. 4). The columnar cells lining the proventricular ducts also presented staining in the cytoplasm, but this staining was more diffuse. The fluorescent staining observed occurred in the areas of the proventriculus that presented lesions. Proventricular glands with no microscopic lesions did not have immunofluorescent staining. Some mononuclear cells also fluoresced, but it was considered an unspecific staining. No staining was observed in tissues from saline-inoculated chickens.

Electron microscopy. Virus-like particles of approximately 120 nm in diameter were present in cells of the proventricular glandular epithelium. They were visualized in the proventriculi from chickens inoculated with Hom. 1 or Hom. 2 examined at 7 dpi (Fig. 5). Virus-like particles were found in vesicles in the cytoplasm of affected cells and budding from the cytoplasm. These particles were pleomorphic and enveloped and resembled coronaviruses. These virus-like particles were observed in all affected proventriculi examined, although they were difficult to find because of the excess of rough endoplasmic reticulum present in these secretory cells. No viruses were found in the nucleus of affected proventricular glandular cells in any of the proventriculi examined. No viruses were found in proventriculi from saline-inoculated chickens.

DISCUSSION

Proventriculitis was successfully reproduced by oral inoculation of commercial and SPF broilers with proventricular homogenates obtained from chickens with proventriculitis. Microscopic lesions consisted of degeneration and necrosis of the glandular epithelium, severe lymphocytic infiltration, and ductal epithelial hyperplasia. This loss of glandular tissue and ductal hyperplasia may result in loss of function of the proventriculus (7). This would explain the poor feed conversion and reduced growth rates reported in some naturally affected chickens with proventriculitis, and the reduced weight gain observed in our homogenate-inoculated SPF chickens. However, the

body weight gain in our commercial chickens was not affected. Bayyari *et al.* (2) found that proventriculitis was produced independently of effects on growth, and a common field observation is that proventriculitis can occur in the best performing flocks (11). This leads us to believe that proventriculitis may or may not be associated with stunting in broilers and that several agents or conditions most likely modify the severity of proventriculitis and its effects, if any, on weight gain. Proventriculitis has been associated with infectious stunting or malabsorption syndrome in chickens (3), but cases of malabsorption syndrome may or may not include proventricular lesions (30). Filterable agents isolated in the Netherlands were originally linked to proventriculitis and caused runting syndrome in broilers (16). Those authors suggested the etiologic involvement of both bacteria and viruses in malabsorption syndrome (16,17). A comparative study of the pathogenesis of five different malabsorption syndrome homogenates from the Netherlands and Germany distinguished the inoculated groups of chickens by their histopathologic lesions: proventriculitis, lesions in the intestine only, or a combination of both (30). Lesions in the small intestine had more impact on weight gain depression than lesions in the proventriculus. In our study, no intestinal lesions were observed in chickens inoculated with the proventricular homogenates. The decreased weight gain observed in the homogenate-inoculated SPF broilers could also be attributed to depression induced by infection with IBDV or IBV present in the homogenates.

Reoviruses have been implicated as a causative agent for concurrent proventricular lesions in some flocks naturally affected with malabsorption syndrome (17), and proventriculitis was reproduced by inoculation of two reovirus isolates from the intestines of birds with malabsorption syndrome (21). In our study, however, no reovirus was isolated from the homogenates, and no reovirus was detected by RT-PCR in any of the inoculated groups. Also none of the chickens seroconverted to this virus, indicating proventriculitis can occur in the absence of reovirus infection.

Mild proventriculitis has also been reproduced experimentally in chickens infected with some isolates of adenovirus (16,18), although this virus has not been consistently isolated from diseased proventriculi. One of the proventricular homogenates used in our study was positive for adenovirus, as were the proventriculi from chickens inoculated with that homogenate. However, the role of this virus in proventriculitis is not clear because the disease still occurred in its absence, and visualization of adenoviral particles in affected

proventricular glands by EM was unsuccessful. Goodwin *et al.* (7) reported the presence of intralesional virions in proventriculi from chicks that had proventriculitis, and suggested a causal relationship between the virus and the lesion in its host. Hexagonal intranuclear virus particles were described and resembled adenovirus or polyomavirus. However, DNA *in situ* hybridization failed to detect adenovirus or polyomavirus nucleic acids. Huff *et al.* (11) also reported the presence of similar virus-like particles in the nuclei of many epithelial cells of the proventriculi of chickens experimentally inoculated with homogenate prepared from the proventriculi of chickens with proventriculitis. The particles, nonenveloped spheres of about 100–200 nm in diameter, appeared hexagonal and were arranged in semiparacrystalline arrays in the nuclei (11). These adenovirus-like particles have not been isolated, so their role, if any, as a causative agent for proventriculitis is not clear. In our study, examination of the proventricular tissues by EM did not reveal viral particles in the nucleus of affected glandular epithelial cells, and IFA staining occurred in the cytoplasm, not the nucleus, of these cells.

IBDV has also been associated with proventriculitis (2,11,20) but its role in this disease is not clear. Both gross and microscopic lesions of the proventriculus have been produced by IBDV challenge in leghorn chickens (29), and vaccination against IBDV has been reported to decrease the incidence of proventriculitis. However, proventriculitis was not produced by inoculation of SPF broilers with different strains of IBDV (22). Both proventricular homogenates used in our study to induce proventriculitis were positive for IBDV by RT-PCR. Proventriculi of commercial broilers inoculated with these homogenates were negative for this virus by RT-PCR, and these birds did not develop lesions or have virus in their bursas. These chickens had antibody titers against IBDV at 7 and 14 dpi and were probably protected against the virus. On the other hand, SPF broilers had lesions in the bursa characteristic of IBDV infection, the virus was detected by RT-PCR in all bursas and some of the proventriculi, and some of the birds seroconverted at 14 dpi. Because proventriculitis was produced in commercial broilers independently of the presence of IBDV, this virus probably is not directly involved in causing proventriculitis.

Both of the proventricular homogenates used in our study to induce proventriculitis were also positive for IBV by RT-PCR, and homogenates produced from the proventriculi of inoculated SPF broilers were also positive by RT-PCR and birds seroconverted to IBV at 14 dpi. These birds also had moderate to severe nephritis, a lesion associated with infection by some strains of IBV. Commercial broilers inoculated with the proventricular homogenates were positive by RT-PCR for IBV and had mild interstitial nephritis at 14 dpi, and IBV was isolated in embryos when inoculated with a filtrate produced from the homogenate prepared from the pooled proventriculi of these birds. These commercial broilers had antibodies against IBV, most likely of maternal origin, detected at both 7 and 14 dpi, which probably offered some protection against the effects of the virus. Electron microscopic examination of proventriculi from homogenate-inoculated chickens revealed the presence of virus-like particles in the cytoplasm of the proventricular glandular epithelial cells. These virus-like particles were 100–120 nm in diameter and morphologically resembled coronavirus. IBV isolates from naturally occurring cases in China have been reported to produce proventricular lesions in infected birds. The serotype of IBV isolated in our study was determined to be Connecticut by RFLP, a serotype that has been isolated also from cecal tonsils and intestine in chickens. The role of IBV in proventriculitis needs to be further explored.

Huff *et al.* (11) reported the isolation of a unique bacterial agent (*Clostridium* sp.) from a proventriculus homogenate that caused

proventriculitis, suggesting bacterial involvement in this syndrome. Those authors concluded that a viral infection, as well as various dietary factors, may facilitate bacterial invasion of the proventriculus, and more than one type of virus may facilitate this disease. In our study, no bacteria were isolated or identified by histopathology or special staining in the proventriculus of affected chickens; however, the role of bacteria should be taken into consideration when studying proventriculitis.

In conclusion, proventriculitis can be transmitted by oral inoculation of homogenates produced from proventriculi of birds with proventriculitis. A specific causative agent was not identified, although most likely it is a virus. The severity of proventriculitis and its effect on weight gain is probably affected by other factors such as concomitant infection with other agents, viral or bacterial, or nutritional factors. Suggested causative viruses of proventriculitis are IBV, IBDV, adenovirus, adenovirus-like virus, and reovirus; however, none of them is found in every case of proventriculitis, and none has been demonstrated to reproduce the disease when inoculated into chickens. In this study, IBV was isolated from affected proventriculus, and coronavirus-like particles were identified by EM. Immunohistochemical staining was observed in the cytoplasm of affected proventricular glandular epithelial cells, the same cells in which the coronavirus-like particles were identified. Our results differ from other investigations in that no adenovirus-like particles were found in the nucleus of proventricular glandular epithelial cells. This leads us to believe that possibly two or more viruses can infect and replicate in the proventriculus, causing a similar disease.

REFERENCES

1. Apple, R. O., J. K. Skeeles, G. E. Houghten, J. N. Beasley, and K. S. Kim. Investigation of a chronic feed passage problem on a broiler farm in northwest Arkansas. *Avian Dis.* 35:422–425. 1991.
2. Bayyari, G. R., W. E. Huff, J. M. Balog, N. C. Rath, and J. N. Beasley. Experimental reproduction of proventriculitis using homogenates of proventricular tissue. *Poult. Sci.* 74:1799–1809. 1995.
3. Bracewell, C. D., and C. J. Randall. The infectious stunting syndrome. *World Poult. Sci. J.* 40:31–37. 1984.
4. Carson, F. L. *Histotechnology. A self instructional text*, 2nd ed. American Society of Clinical Pathologists, Chicago. 1997.
5. Cavanagh, D., and S. A. Naqi. Infectious bronchitis. In: *Diseases of poultry*, 10th ed. H. J. B. W. Calnek, C. W. Beard, L. R. McDougald, and Y. M. Saif, eds. Iowa State University Press, Ames, IA. pp. 511–526. 1997.
6. Genta, R., G. O. Robason, and D. Y. Graham. Modified *Helicobacter pylori* and gastric morphology. *Human Pathol. J.* 25:221–226. 1994.
7. Goodwin, M. A., S. Hafner, D. I. Bounous, K. S. Latimer, E. C. Player, F. D. Niagro, R. P. Campagnoli, and J. Brown. Viral proventriculitis in chickens. *Avian Pathol.* 25:269–279. 1996.
8. Hajna, A. A., and S. R. Damon. New enrichment and plating for the isolation of *Salmonella* and *Shigella* organisms. *Appl. Microbiol.* 4: 341–345. 1956.
9. Henderson, G. M., F. M. Gulland, and C. M. Hawkey. Haematological findings in budgerigars with megabacterium and *Trichomonas* infections associated with 'going light.' *Vet. Rec.* 123:492–494. 1988.
10. Huchermeyer, F. W., M. M. Henton, and R. H. Keffen. High mortality associated with megabacteriosis of proventriculus and gizzard in ostrich chicks. *Vet. Rec.* 133:143–144. 1993.
11. Huff, G. R., Q. Zheng, L. A. Newberry, W. E. Huff, J. M. Balog, N. C. Rath, K. S. Kim, E. M. Martin, S. C. Goeke, and J. K. Skeeles. Viral and bacterial agents associated with experimental transmission of infectious proventriculitis of broiler chickens. *Avian Dis.* 45:828–843. 2001.
12. Huff, W. E., J. M. Balog, G. R. Bayyari, and N. C. Rath. The effect of Mycocurb, propionic acid, and calcium propionate on the intestinal strength of broiler chickens. *Poult. Sci.* 73:1352–1356. 1994.

13. Jackwood, M. W., N. M. Yousef, and D. A. Hilt. Further development and use of a molecular serotype identification test for infectious bronchitis virus. *Avian Dis.* 41:105–110. 1997.
14. Karaca, K., S. A. Naqi, P. Palukaitis, and B. Lucio. Serological and molecular characterization of three enteric isolates of infectious bronchitis virus of chickens. *Avian Dis.* 34:899–904. 1990.
15. Kho, C. L., M. L. Mohd-Azmi, S. S. Arshad, and K. Yusoff. Performance of an RT-nested PCR ELISA for detection of Newcastle disease virus. *J. Virol. Methods* 86:71–83. 2000.
16. Kouwenhoven, B., F. G. Davelar, and J. Van Walsum. Infectious proventriculitis causing runting in broilers. *Avian Pathol.* 7:183–187. 1978.
17. Kouwenhoven, B., M. Vertomment, and E. Goren. Investigations into the role of reovirus in the malabsorption syndrome. *Avian Pathol.* 17: 879–892. 1988.
18. Lenz, S. D., F. J. Hoerr, A. C. Ellis, M. A. Toivio-Kinnucan, and M. Yu. Gastrointestinal pathogenicity of adenoviruses and reoviruses isolated from broiler chickens in Alabama. *J. Vet. Diagn. Invest.* 10:145–151. 1998.
19. Mutlu, O. F., S. Seckin, K. Ravelhofer, R. A. Hildebrand, and F. Grimm. [Proventriculitis in domestic fowl (*Gallus gallus* var. dom. L., 1758) caused by megabacteria]. *Tierarztl. Prax. Ausg. G Grosstiere Nutztiere* 25: 460–462. 1997.
20. Newberry, L. A. Determination of the role of standard and variant strains of infectious bursal disease virus in the induction of viral proventriculitis and enteric disease in chickens. Ph.D. Dissertation. University of Arkansas, Fayetteville, AR. 1996.
21. Page, R. K., O. J. Fletcher, G. N. Rowland, D. Gaudry, and P. Villegas. Malabsorption syndrome in broiler chickens. *Avian Dis.* 26: 618–624. 1982.
22. Pantin-Jackwood, M. J., and T. P. Brown. Infectious bursal disease virus and proventriculitis in broiler chickens. *Avian Dis.* 47: 681–690. 2003.
23. Pantin-Jackwood, M. J., T. P. Brown, and G. R. Huff. Proventriculitis in broiler chickens: immunohistochemical characterization of the lymphocytes infiltrating the proventricular glands. *Vet. Pathol.* 41:641–648. 2004.
24. Pantin-Jackwood, M. J., T. P. Brown, Y. Kim, and G. R. Huff. Proventriculitis in broiler chickens: effects of immunosuppression. *Avian Dis.* 48:300–316. 2004.
25. Phalen, D. N., and R. P. Moore. Experimental infection of white-leghorn cockerels with *Macrorhabdus ornithogaster* (megabacterium). *Avian Dis.* 47:254–260. 2003.
26. Raue, R. and M. Hess. Hexon based PCR's combined with restriction enzyme analysis for rapid detection and differentiation of fowl adenovirus and egg drop syndrome virus. *J. Virol. Methods* 73:211–217. 1998.
27. Senne, D. A. Virus propagation in embryonating eggs. In: *A laboratory manual for the isolation and identification of avian pathogens*, 4th ed. J. R. G. D. E. Swayne, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 235–247. 1998.
28. Shapiro, F., and I. Nir. Stunting syndrome in broilers: physical, physiological, and behavioral aspects. *Poult. Sci.* 74:33–44. 1995.
29. Skeeles, J. K., L. A. Newberry, J. N. Beasley, and B. A. Hopkins. Histologic comparison of lesions induced in the proventriculus and other areas of the intestinal tract of chickens experimentally infected with both classic and variant strains of infectious bursal disease virus. *Poult. Sci.* 77(Suppl.):133. 1998.
30. Songserm, T., J. M. Pol, D. van Roozelaar, G. L. Kok, F. Wagenaar, and A. A. ter Huurne. A comparative study of the pathogenesis of malabsorption syndrome in broilers. *Avian Dis.* 44:556–567. 2000.
31. Xie, Z., A. A. Fadl, T. Girshick, and M. I. Khan. Amplification of avian reovirus RNA using the reverse transcriptase-polymerase chain reaction. *Avian Dis.* 41:654–660. 1997.
32. Yu, L., Y. Jiang, S. Low, Z. Wang, S. J. Nam, W. Liu, and J. Kwangac. Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. *Avian Dis.* 45:416–424. 2001.

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